

## Article

# Distinguishing Long-Discussed Cryptic Species of the Epibiotic Goose-Neck Barnacle of the Genus *Conchoderma* (Thoracicalcareia: Lepadidae) with Integrative Taxonomy

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**Abstract:** Naked goose neck barnacles *Conchoderma* can grow on a wide variety of marine organisms. The taxonomic status of two of its species—*C. virgatum* and *C. hunteri*—are currently controversial. Some studies suggest that *C. hunteri* is a subspecies, variety or growth forms of *C. virgatum*, because both have great morphological variations, but other studies consider *C. hunteri* and *C. virgatum* to be distinct species. The present study examines the morphology and sequence divergence of the COI gene in *C. virgatum*, *C. hunteri* and other closely related species. There are consistent morphological differences between *C. virgatum* and *C. hunteri* in the tergum, carina and fifth teeth of the mandible. Phylogenetic analysis based on the divergence in the COI gene revealed that *C. virgatum* and *C. hunteri* form sister clades with high bootstrap values. The K2P distances within *C. hunteri* and *C. virgatum* are  $0.034 \pm 0.008$  and  $0.002 \pm 0.001$  for the COI sequences, respectively. The K2P distance between *C. hunteri* and *C. virgatum* is  $0.097 \pm 0.016$ . Morphological and molecular evidence confirm that *C. hunteri* is a valid species.

**Keywords:** *Conchoderma*; taxonomy; barnacles; epibiosis; COI



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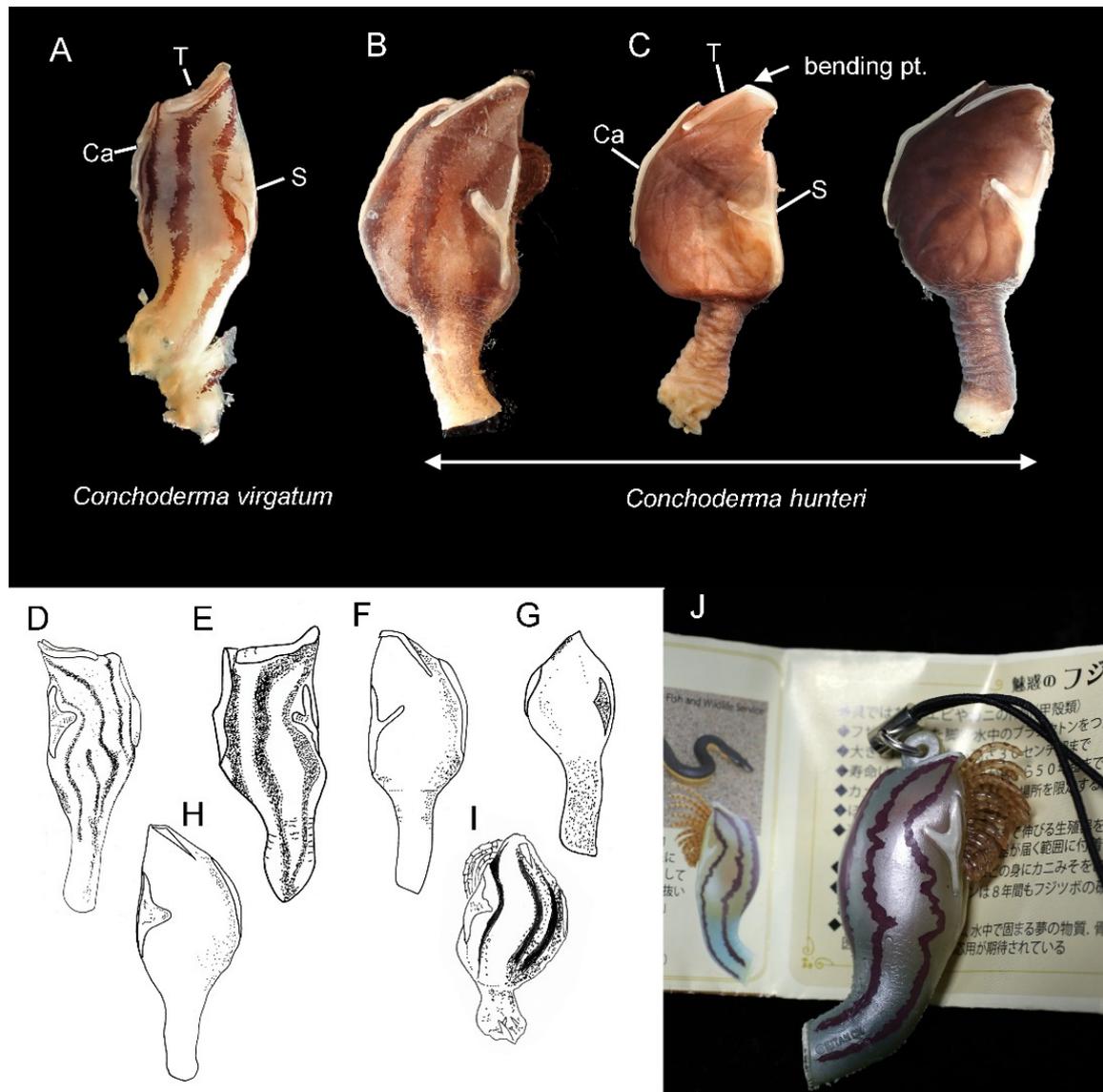
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## 1. Introduction

Marine biologists have been interested in the naked goose neck barnacles *Conchoderma* (Lepadidae; latest classification see [1]) for a long time, because species in the genus live on a variety of marine organisms, including copepods, isopods, sea snakes, whales, fishes, crabs and sea turtles [2–6]. The capitulum of *Conchoderma* has highly reduced shell plates, including the tergum, scutum and carina [7,8]. At present, *Conchoderma* comprises four or five species: *C. auritum*, *C. virgatum* (Spengler, 1790) [9], *C. hunteri* (Owen, 1830) [10], *C. chelonophilum* (Leach, 1818) [11] and *C. indicum* (Daniel, 1953) [12]; however, the taxonomic status of *C. virgatum* and *C. hunteri* is controversial because they have great morphological variations in their tergum, scutum and capitulum [13,14].

*C. virgatum* has three longitudinal bands on its capitulum [7,15] (Figure 1). *C. hunteri* has a brown colored capitulum and occasionally with faint vertical bands [7] (Figure 1). The number of filamentary appendages, morphology of cirri and mouth parts are similar between *C. hunteri* and *C. virgatum* [15]. Darwin [7] described *C. hunteri* and compared it to *C. virgatum* with the comment (page 155) “I have very great doubt whether I have acted rightly in considering this as a species.” However, based on the variation in the morphology of their tergum, Darwin [7] concluded *C. hunteri* and *C. virgatum* are different species. Hoek [16] considered *C. hunteri* to be morphologically similar to *C. virgatum chelonophilum* and *C. hunteri* to be a subspecies of *C. virgatum*. Annandale [17] followed Hoek’s subspecies designation for *C. hunteri*. Hiro [13] doubted that *C. hunteri* is a sub-species or variety of *C. virgatum* or a juvenile form of *C. virgatum*, because previous records of *C. hunteri* identified the species to be smaller than *C. virgatum*. Finally, Hiro [13] considered *C. hunteri* to be a subspecies of *C. virgatum*. Zevina [18] supported *C. hunteri* and *C. virgatum* as

conspecific. In contrast, Liu and Ren [8,19] and Yamato et al. [4] considered *C. virgatum* and *C. hunteri* to be different species. Yorisue et al. [5] doubted the taxonomic status of *C. virgatum* and *C. hunteri* when examining the diversity of *Conchoderma* in crabs in Japan and called the two species *Conchoderma* sp. in their study.



**Figure 1.** (A). *Conchoderma virgatum*, showing the capitulum with three longitudinal dark bands. (B). *Conchoderma hunteri*, specimen with faint dark bands on the capitulum. (C). *Conchoderma hunteri* with pale brown (left) and darker brown (right) capitulum. Note tergum bends at the distal 1/4 portion. (D). *C. virgatum*, redrawn from Darwin (1851) [7]. (E). *C. virgatum* redrawn from Pilsbry (1907) [15]. (F). *C. hunteri*, redrawn from Darwin (1851) [7]. (G). *C. indicum* redrawn from Daniel (1953) [12]. (H). *C. chenolophilum* redrawn from Darwin (1851) [7]. (I). *C. chenolophilum* redrawn from Hoek (1883) [16]. (J). A Japanese plastic key chain of *C. virgatum* and with its biological descriptions. Ca—Carina, S—scutum and T—tergum.

To ascertain the taxonomic status of *C. hunteri* and *C. virgatum*, it is essential to conduct further detailed morphological comparisons and molecular studies on their sequence divergences. The present study examines the morphology and sequence divergence in the COI gene of *C. hunteri* and *C. virgatum* to determine the taxonomic status of *C. hunteri* and *C. virgatum*.

## 2. Materials and Methods

### 2.1. Sample Collections

A colony of *Conchoderma virgatum* was collected in Kenting, Taiwan. Several colonies of *C. hunteri* were collected in Taiwan and the Philippines (see materials examined below for the collection details).

### 2.2. Morphological Examination

The size (mm) of the stalked barnacle was measured as capitular length (CL) by capitular width (CW). Specimens were dissected under a Leica stereomicroscope, mouth parts, setal-types of cirri and dwarf males were examined under a Zeiss Axioplan compound microscope installed with objectives APO 40× and APO 20× lenses.

### 2.3. Molecular Analysis

The mitochondrial COI gene (cytochrome *c* oxidase subunit I) was sequenced from *Conchoderma* specimens. DNA was extracted from muscle tissue using the commercial QIAamp Tissue Kit (QIAGEN). Partial sequences of the mitochondrial COI gene were amplified using a PCR universal primer set (LCO-1490/HCO-2198) for DNA barcoding [20]. Polymerase chain reaction (PCR) was performed using 25 µL of a solution containing 12.5 µL of Taq 2× Tools Taq Master Mix (AMPLIQON), 7.5 µL of deuterium-depleted water, 1 µL of each primer (5 µM) and 3 µL of the DNA template. The thermal regime entailed initialization for 4 min at 94 °C, denaturation using 35 cycles for 0.5 min at 94 °C, 0.5 min at 50 °C and 1 min at 72 °C, elongation for 10 min at 72 °C and a final extension at 12 °C. The PCR products were observed on 1.5% agarose gel, and the most intense products were used for Sanger sequencing. Successfully amplified PCR products were purified and sequenced using Genomics BioSci & Tech Ltd. Sequences were deposited in National Center for Biotechnology Information (NCBI) GenBank (<https://www.ncbi.nlm.nih.gov/>) and Barcode of Life Data system (BOLD, <https://www.boldsystems.org/>).

### 2.4. Phylogenetic Analysis

Eleven COI sequences of species in Lepadidae (KU204318, GU993634, MT563438, MT563441 and KU204353); Heteralepadidae (EU884174 and EU8841690); Poecilasmatidae (KC138499, KC138498 and EU884173) and Scalpellidae (KT208441) were downloaded from NCBI GenBank and included in the dataset. We also included 1 COI sequence of *Lepas* (ON938319) and *Oxynaspis* (ON938312) from our specimen collection. All the sequences were aligned with Clustal W in MEGAX [20–22] (See Supplementary Materials for the aligned sequences).

Neighbor-joining (NJ) and maximum likelihood (ML) tree were inferred by using MEGAX [23]. A Bayesian inference (BI) analysis was also performed using MrBayes 3.2.7 [24]. For the NJ and ML tree, the T92 + G model was selected as the best substitution model according to the model test in MEGAX with 1000 bootstrap replicates [25]. The BI analysis was conducted with  $1 \times 10^6$  generations of 5 MCMC chains. Trees were saved every 1000 generations, and the first 25% of trees were discarded as burn-in.

Assemble species with automatic partitioning analysis (ASAP) (<https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html>, accessed on 11 July 2022) [26], Poisson tree processes (PTP) model (<https://species.h-its.org/ptp/>, accessed on 11 July 2022) [27] and Generalized Mixed Yule Coalescent (GMYC) method [28,29] were used to confirm the species delimitation. For ASAP, Juke-Cantor model (JC69), Kimura 2-parameter model (K2P) and Simple Distance (*p*-distances) were used, respectively. The PTP method was performed with the ML tree (see above) for 500,000 MCMC generations and saved every 500 generations. The first 25% of generations were discarded as burn-in. For GMYC method, phylogenetic tree was inferred by using BEAST v. 1.10.4 [30] with Yule process. HKY + G was selected as substitution model based on the result of model test in MEGAX. Three MCMC chains were run for  $1 \times 10^6$  generations, and sampled every 1000 generations. The run convergence was visually verified in Tracer v. 1.7.2 [31], and the effective sample size

(ESS) of all parameters was >200. The tree files from the 3 runs were combined and discarded the first 25% as burn-in using LogCombiner v. 1.10.4 (BEAST package) and assessed using TreeAnnotator v. 1.10.4 (BEAST package). After ultrametric tree was constructed, the GMYC method was performed in R software v. 4.1.2 using the “splits” package (Species Limits by Threshold Statistics) v. 1.0-20 [32]. Kimura 2-parameter (K2P) distances of COI sequences within/between species were also calculated using MEGAX [33] (Table 1).

**Table 1.** Specimen information and GenBank accession numbers for DNA sequences.

Species	Specimen Voucher	Locality	GenBank Accession Numbers	Reference
<i>Lepas anserifera</i>	KU204318	Apra Harbor, Guam	KU204318	[34]
	Lep_sp_001	Taitung, Taiwan	ON938319	Present study
<i>Scalpellum scalpellum</i>	KT208441	North Sea, Taiwan	KT208441	[35]
<i>Octolasmis cor</i>	KC138499	Pingtung, Taiwan	KC138499	[36]
<i>Octolasmis angulata</i>	KC138498	Ha Long Bay, Vietnam	KC138498	Unpublished
<i>Lepas australis</i>	GU993634	Chile	GU993634	Unpublished
<i>Paralepas laxis</i>	EU884174	-	EU884174	
<i>Octolasmis orthogonia</i>	EU884173	-	EU884173	[37]
<i>Heteralepas japonica</i>	EU884169	-	EU884169	
<i>Oxynaspis</i> sp.	CD_3257_1	Madagascar	ON938312	Present study
<i>Conchoderma hunteri</i>	Con_h_01	Panglao Island, Philippines	KF484213	
	Con_h_02		KC138463	
	Con_h_03	Penghu, Taiwan	ON938315	[38]
	Con_h_04		ON938315	
<i>Conchoderma virgatum</i>	Con_v_02	Pingtung, Taiwan	KC138464	
	Con_sp_01	Kagoshima, Japan	ON938317	[36]
<i>Conchoderma auritum</i>	MT563438	-	MT563438	
	MT563441	-	MT563441	Unpublished
	KU204353	Portland, USA	KU204353	[34]

### 3. Results

#### 3.1. Taxonomy

**Superorder Thoracicalcareia** Gale, 2015 [39].

**Order Scalpellomorpha** Buckeridge and Newman, 2006 [40].

**Family Lepadidae** Darwin, 1851 [7].

**Genus *Conchoderma*** von Olfers, 1814 [41].

*Conchoderma virgatum* (Figure 1A, 2–6).

*Lepas virgata* Spengler 1790: Figure [9].

*Conchoderma virgata* Darwin 1851: 146, pl. 3, Figure 4 [7].—Zevina 1980: 692 [42].

*Conchoderma virgatum* Hoek 1883: 55 [16].—Gruvel 1905: 144, Figure 169 [43].—Gruvel 1920: 38 [44].—Pilsbry 1907: 99, pl. 9, Figure 1 [15].—Annandale 1909: 80 [17].—Stebbing 1910: 566 [45].—Jennings 1918: 59 [46].—Nilsson-Cantell 1921: 242 [47].—Nilsson-Cantell 1928: 16, Figure 7 [48].—Nilsson-Cantell 1930: 251, pl. 1, Figure 4 [49].—Nilsson-Cantell 1939: 236 [50].—Broch 1924: 58, Figure 20 [51].—Barnard 1924: 61 [52].—Krüger 1927: 13 [53].—Hiro 1936: 623, Figure 2 [54].—Hiro 1937a: 402, Figure 5 [13].—Hiro 1937b: 62, Figure 53 [55].—Hiro 1939a: 205 [56].—Hiro 1939b: 248 [57].—Utinomi 1970: 341 [58].—Balakrishnan 1969: 102, pl. 1 [59].—Newman and Ross 1971: 35, Figure 11, pl. 5E [2].—Hastings 1972: 274 [3].—Williams 1978: 109, pl. 1 [60].—Munroe and Limpus 1979: 198 [61].—Foster and Willan 1979: Figure 3d [62].—Zevina 1982: 27, Figure 16 [18].—Liu and Ren 1985: 225, Figure 23, 24, pl. 6: 6–11 [19].—Eckert and Eckert 1987: 682 [63].—Yamato et al. 1996: 338, Figure 2 (left) [4].—Liu and Ren 2007: 199, Figure 82 [8].—Alonso et al. 2010: 167, Figure 2 [64].

**Materials examined:** Xiangjiaowan, Kenting, Pingtung Co., June. 2003: 9 specimens (CL 4.26~9.35 mm, CW 2.21~6.01 mm) (CEL-BB79).

**Diagnosis:** Capitulum with three or more dark longitudinal bands. Tergum not bended, mandible with five distinct large teeth.

**Description:**

**Capitulum.** Capitulum flattened, light blue in color when alive, five extremely reduced opercular plates (a single carina, paired scutum and tergum; Figure 1A), three dark longitudinal purplish brown stripes extending from top of capitulum to the base of peduncle (Figure 1A). Head region of capitulum rectangular or trapezium-shaped (Figure 1A). Tergum straight, distal end pointed, proximal end blunted (Figure 1A). Carina sinus and short, tapers to each end. Scutum tri-lobed and y-shaped, upper and lower lobes narrow, medial lobe wide and blunt triangular-shaped (Figure 1A).

**Cirri.** Cirrus I, anterior rami six segmented, posterior rami eight segmented, segments protuberant (except the first-third distal segments) with height 3 times greater than width (Figure 2A–D), bearing dense simple and serrulate-type setae (Figure 2B–D). Base of cirrus I with two pairs of filamentary appendages (Figure 2A). Cirrus II, posterior rami 10-segmented, anterior rami 8-segmented, up to last proximal 5 segments protuberant, with denser serrulate setae (Figure 2E–G). Intermediate segments of cirrus II with two pairs of long serrulate setae and two pairs of short simple setae (Figure 2H). Base of cirrus II without filamentary appendages. Cirrus III, posterior ramus 10 segmented, anterior ramus broken (Figure 3A), intermediate segment bears two pairs of long and two pairs of short simple setae (Figure 3B,D). Distal segments with robust simple setae (Figure 3C). Cirrus IV–VI similar in morphology, both anterior and posterior ramus similar in length (Figure 3E–H and Figure 4A–F). Intermediate segments of cirrus III to VI bears 2 pairs of long simple setae and one pair of short simple setae (Figures 3H and 4B,F). Base of each cirrus III–VI bear one pair of filamentary appendages. Penis thick and short, without basidorsal point, being 1/4 length of the cirrus VI (Figure 4G,H).

**Trophi.** Maxilla circular, with simple and serrulate-type setae on exterior margin (Figure 5A–C). Maxillule with one large setae at upper margin (Figure 5D,E), cutting edge divided into three zig-zagged portions (Figure 5D), each portion three to four large setae (Figure 5F,G). Mandibles penta-dentoid (Figure 6A); first teeth large, robust and sharp, separated from the remainders (Figure 6B). Second and fifth teeth sharp, large and robust (Figure 6B,C). Teeth surfaces with small sharp triangular setae. Inferior angle with two small spines (Figure 6D). Mandibular palp elongated and with serrulate setae on tip and outer margin (Figure 6E,F). Labrum not bullate, with a small, concaved notch; a single array of small sharp teeth at the notch (Figure 6G,H).

**Distribution:** Cosmopolitan. On surfaces of buoys, snakes, sea turtles, copepods, isopods, fishes.

*Conchoderma hunter* Figure 1B,C, 7–12.

*Cineras hunteri* Owen 1830: 71 [10]

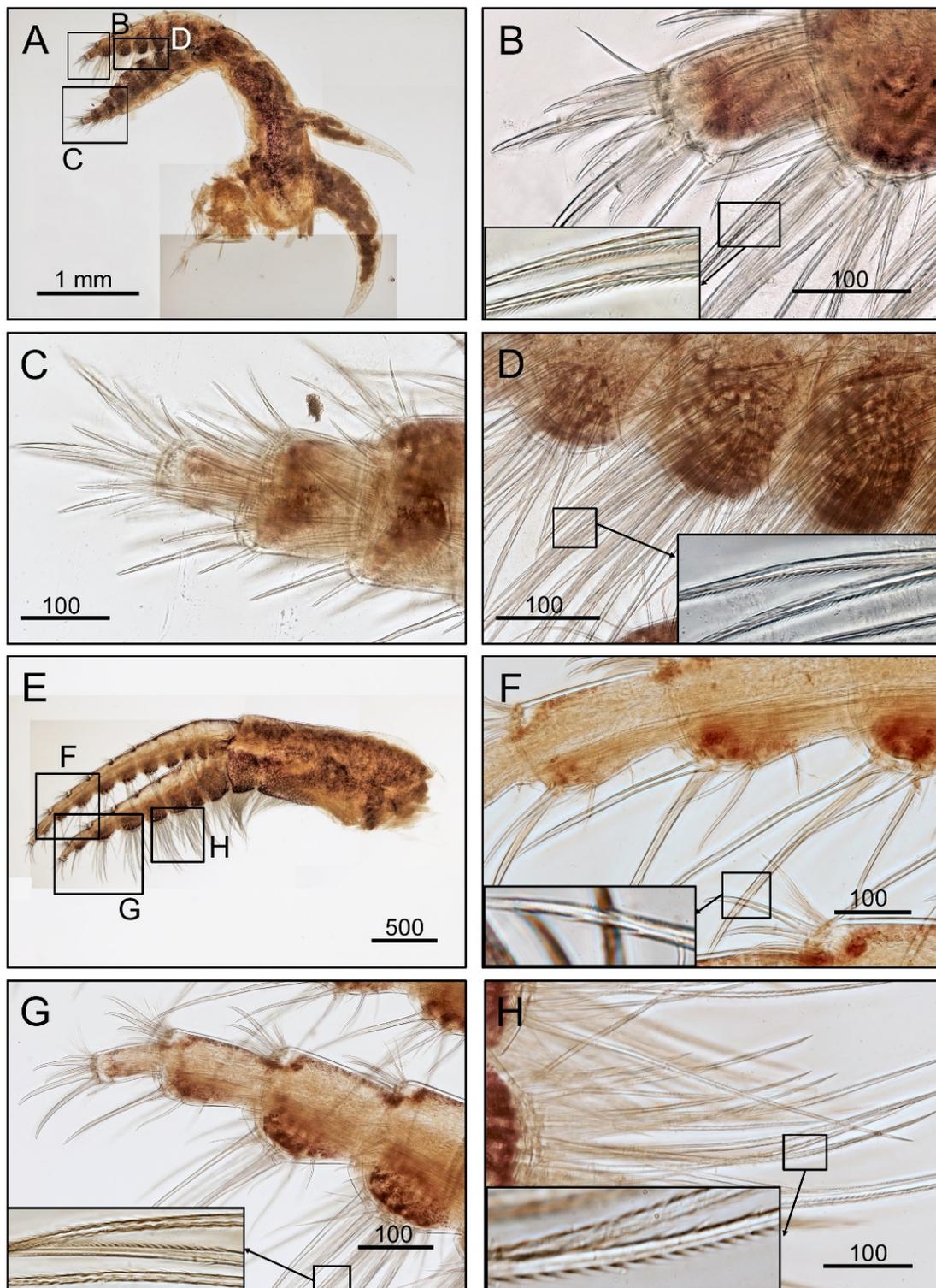
*Conchoderma hunteri* Darwin 1851: 153, pl. 3, Figure 3 [7].—Gruvel 1905: 145, Figure 169 [43].—Barnard 1955: 247 [65].—Liu and Ren 1985: 227, Figure 25, pl. 6, 12–16 [19].—Yamato et al. 1996: 338, Figure 2 (right) [4].—Liu and Ren 2007: 200, Figure 83 [8].

*Conchoderma virgatum* var. *hunteri* Annandale 1909: 82 [17].—Krüger 1911: 26, pl. 3, Figures 20–22 [66].

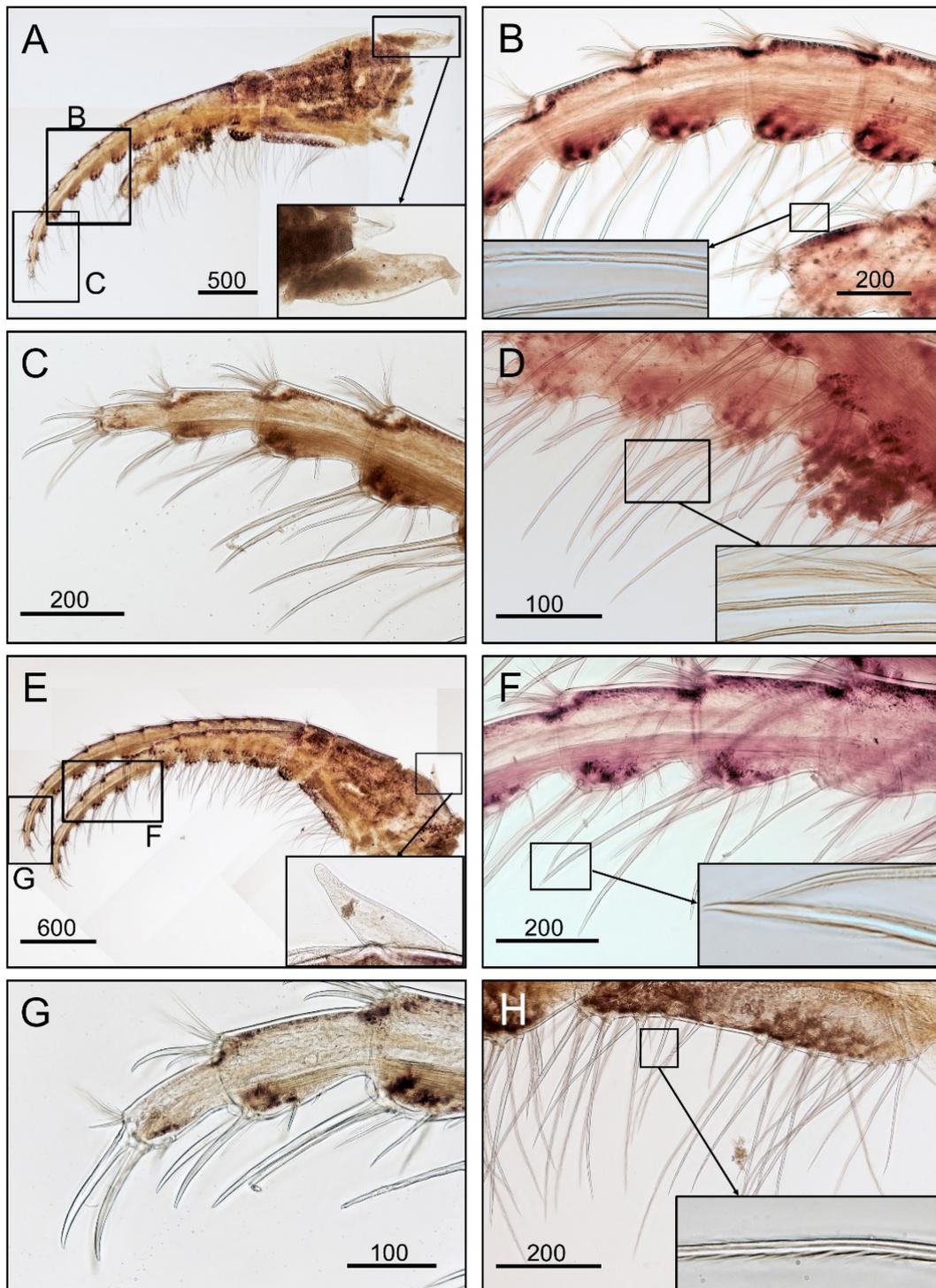
*Conchoderma virgatum* var. *japonica* Krüger 1911: 27, pl. 3, Figure 23 [66].

*Conchoderma virgatum* forma *hunteri* Broch 1931: 28 [67].—Nilsson-Cantell 1938: 27 [68].

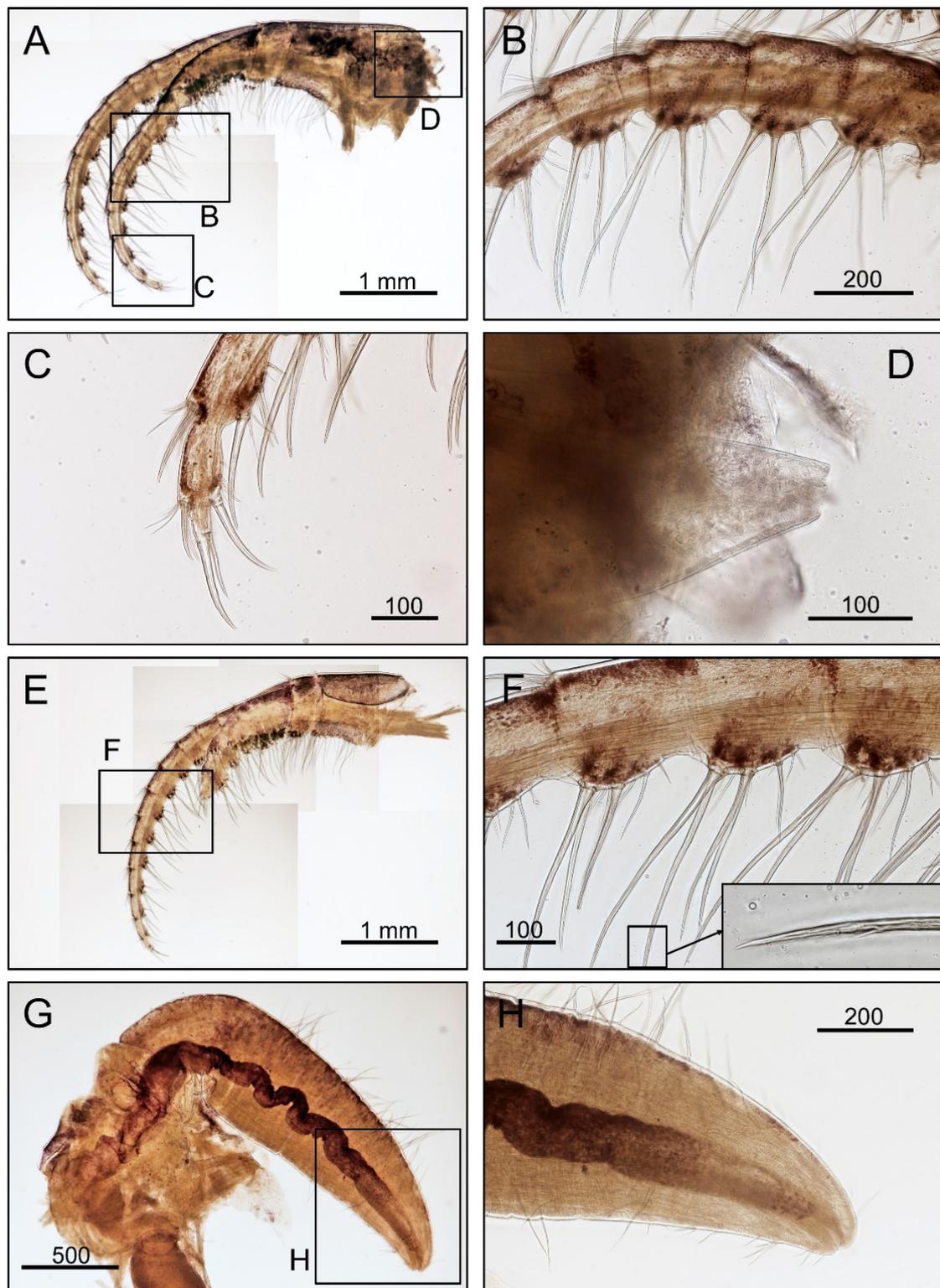
*Conchoderma virgatum hunteri* Hiro 1937a: 402, Figure 6 [13].—Hiro 1937b: 63, Figure 53 [55].—Hiro 1939a: 205 [56].—Utinomi 1968: 167 [69].—Utinomi 1970: 341 [58].—Gordon 1970: 21, Figure 6 [70].—Dong et al. 1980: 125 [71].



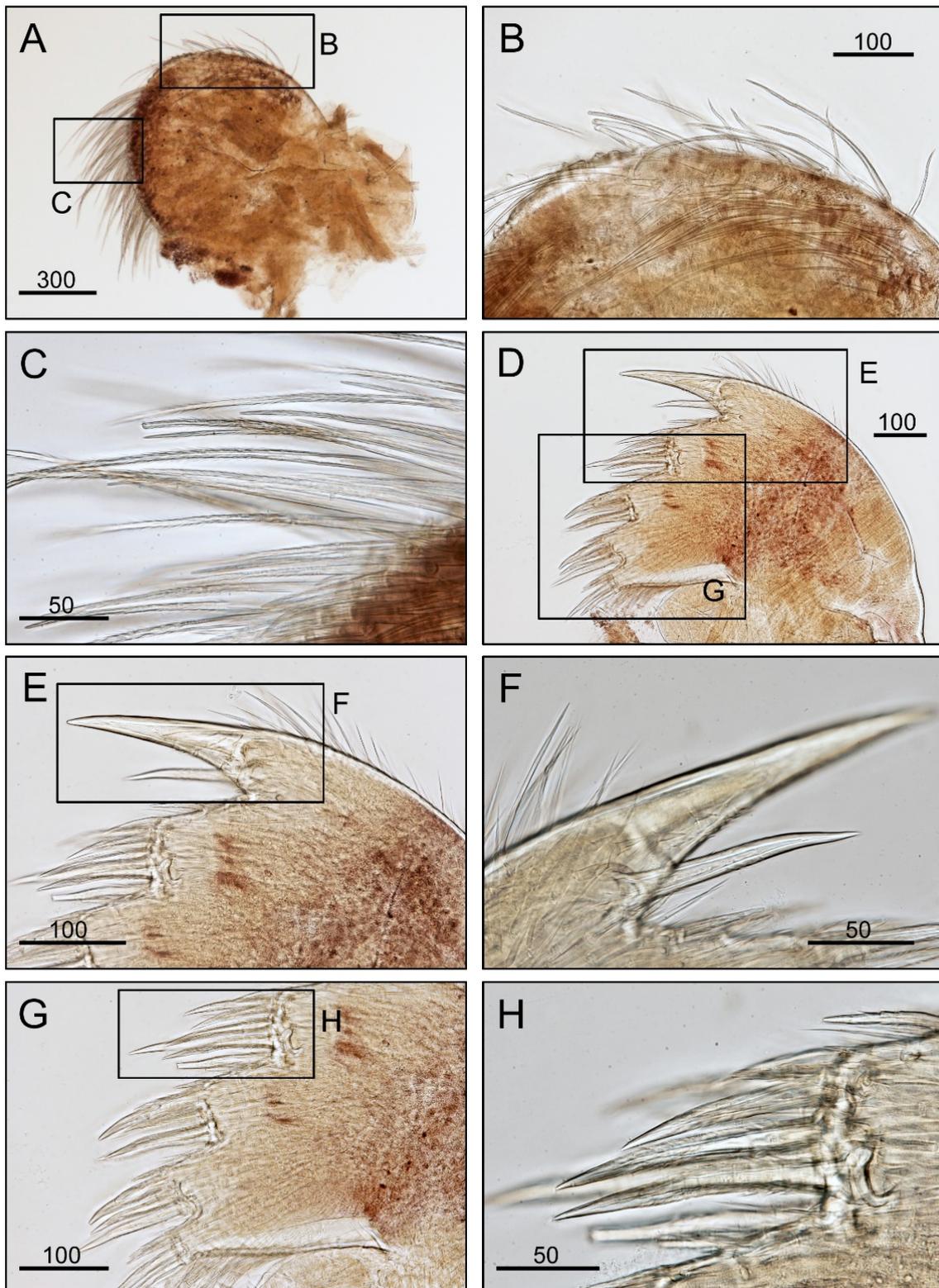
**Figure 2.** *Conchoderma virgatum*. (A) Cirrus I, showing two filamentary appendages at base region. (B) Posterior ramus, showing serrulate setae. (C) Anterior ramus, showing serrulate setae. (D) Pro-tuberant segment of cirrus I, showing serrulate setae. (E) Cirrus II. (F) Posterior ramus showing serrulate setae. (G) Anterior ramus, showing serrulate setae. (H) Intermediate segment of anterior ramus, showing serrulate setae. Scale bars in  $\mu\text{m}$ .



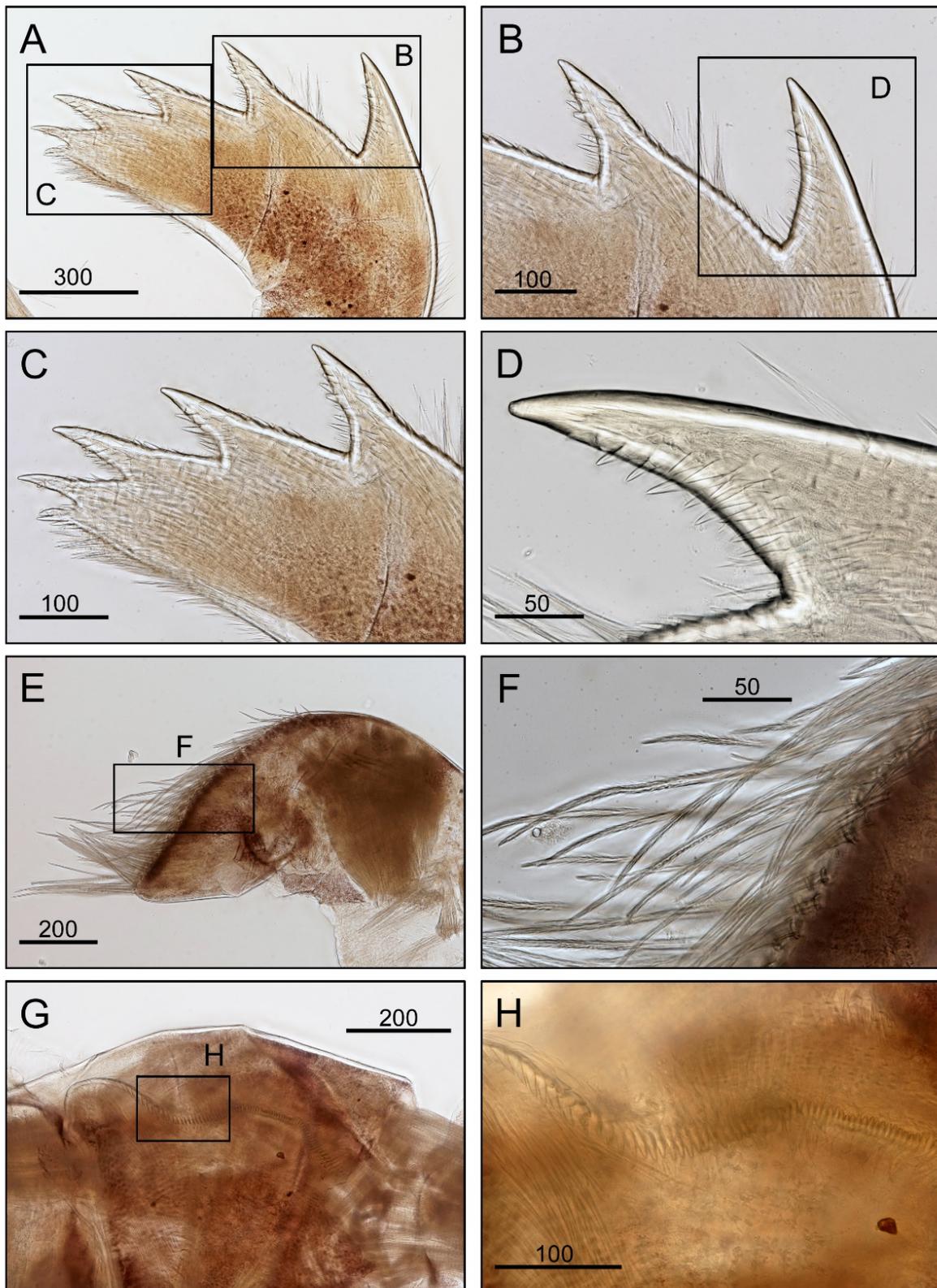
**Figure 3.** *Conchoderma virgatum*. (A) Cirrus III, note single filamentary appendage at base, anterior ramus broken. (B) Intermediate segments of posterior ramus, showing serrulate setae. (C) Distal segment of posterior ramus. (D) Magnified segment of anterior ramus of cirrus III, showing serrulate setae. (E) Cirrus IV, showing small filamentary appendage at base. (F) Simple-type setae at cirrus IV. (G) Distal end of posterior ramus of cirrus IV, showing short robust simple setae. (H) Intermediate segment of cirrus IV, showing serrulate setae. Scale bars in  $\mu\text{m}$ .



**Figure 4.** *Conchoderma virgatum*. (A) Cirrus V, with single small filamentary appendage at base, highlighted in D. (B) Intermediate segment of cirrus V. (C) Distal segment of cirrus V, showing robust simple setae. (D) Filamentary appendage at based. (E) Cirrus VI. Note one ramus was broken. (F) Serrulate setae at intermediate segment of cirrus VI. (G) Penis. (H) Apex of penis. Scale bars in  $\mu\text{m}$ .



**Figure 5.** *Conchoderma virgatum*. (A) Maxilla. (B) Simple setae at outer margin of maxilla. (C) Serrulate setae at inner margin of maxilla. (D) Maxillule showing three zig-zag regions at the cutting edge. (E) Large spine of maxillule. (F) Simple setae at the base of large spine of maxillule. (G) Cutting edge of maxillule. (H) Serrulate-type setae at the cutting edge of maxillule. Scale bars in  $\mu\text{m}$ .



**Figure 6.** *Conchoderma virgatum*. (A) Mandible showing five teeth. (B) First and second teeth of mandible. (C) Third, fourth, and fifth teeth of mandible. (D) First teeth of mandible showing a surface with dense simple setae. (E) Mandibulatory palp. (F) Serrulate setae at the margin of palp. (G) Labrum. (H) Cutting edge of labrum showing small teeth. Scale bars in  $\mu\text{m}$ .

**Materials examined:** CP2362, Panglao Island, the Philippines, 8°56.5' N, 123°32.7' E, 679–684 m, 6 May 2005: 30 specimens (CL 10.56~22.64 mm, CW 6.9~10.56 mm). Tiaoshi, Kenting, Pingtung Co., 2 Feb. 2007: 7 specimens (CL 14.86~19.4 mm, CW 9.29~12.52 mm) (CEL-BB94).

**Diagnosis:** Capitulum dark brown or purplish brown, tergum bended at 1/3 distal portion. Mandible with five teeth, the fifth teeth small.

Description:

**Capitulum.** Capitulum flattened, dark brown or purplish brown in color, opercular plates extremely reduced, occasion with dark stripes extending from top of capitulum to the peduncle and base (Figure 1B,C). Upper margin of capitulum pointed. Tergum bended at one fourth of its distal length (Figure 1B), with one end pointed and the other end blunted. Carina long, overlapping with tergum at 1/4 distal region. Scutum tri-lobed (Figure 1B,C).

**Cirri.** Cirrus I, posterior and anterior rami eight segmented, segments protuberant (except the first-third distal segments) with height three times greater than width (Figure 7A–D), bearing dense simple and serrulate-type setae (Figure 7B,C). Base of cirrus I with two pairs of filamentary appendages (Figure 7A). Cirrus II, posterior rami 12-segmented, anterior rami 11-segmented, up to the last proximal 5 segments protuberant, with denser serrulate setae (Figure 7E–G). Intermediate segments of cirrus II with dense serrulate and simple setae (Figure 7H). Base of cirrus II without filamentary appendages. Cirrus III, posterior ramus 16-segmented, anterior ramus 15-segmented (Figure 8A), intermediate segment bears two pairs of long and two pairs of short simple setae (Figure 8B,D). Distal segments with robust simple setae (Figure 8C). Cirrus IV–VI similar in morphology, both anterior and posterior ramus similar in length (Figures 8E–H and 9A–F). Intermediate segments of cirrus III to VI bears two pairs of long simple setae and one pair of short simple setae (Figures 8H and 9B,F). Base of each cirrus III–VI bear one pair of filamentary appendages. Penis thick and short, without basidorsal point, being 1/4 length of the cirrus VI (Figure 9G,H).

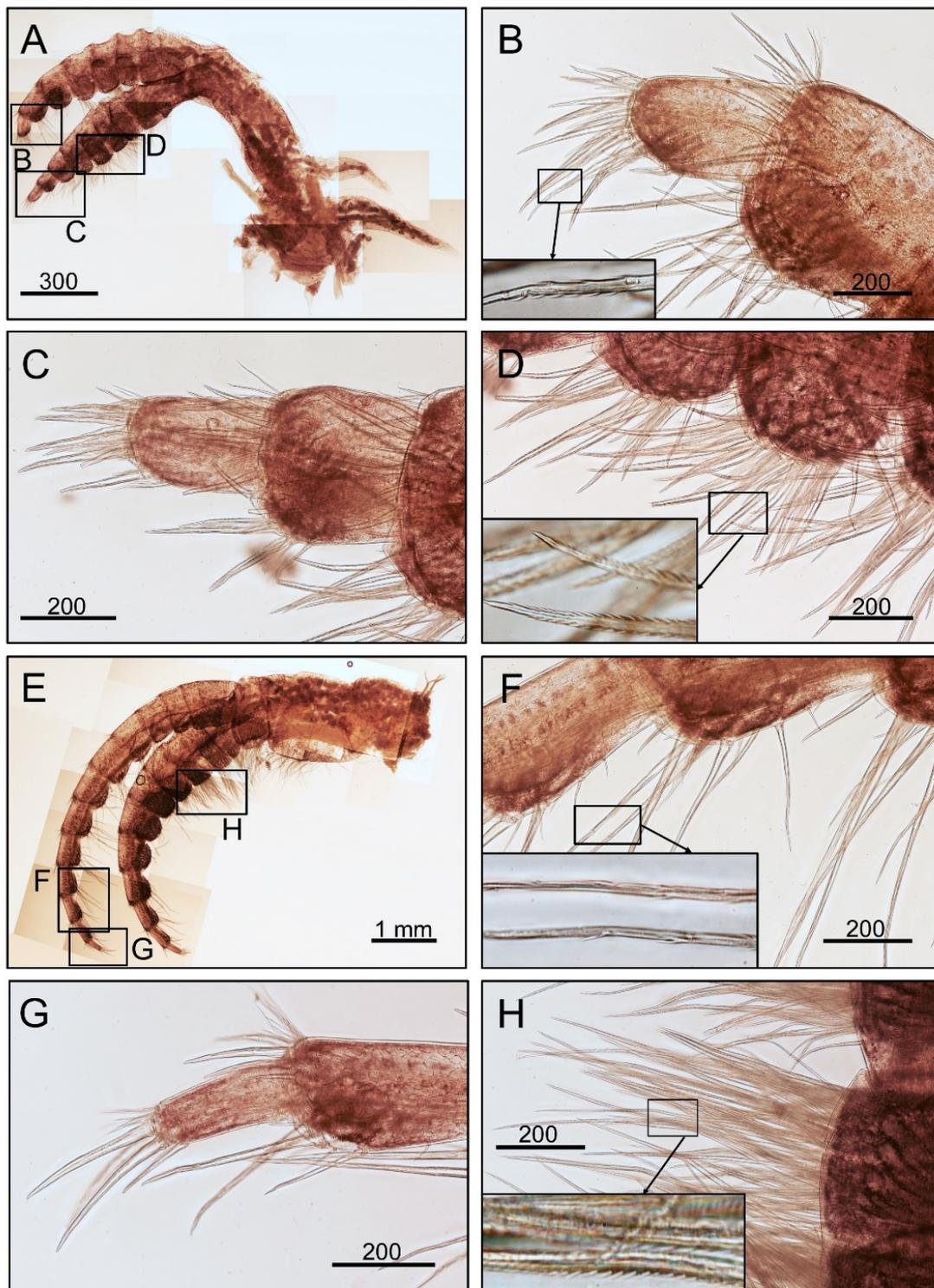
**Trophi.** Maxilla circular, with simple and serrulate-type setae on exterior margin (Figure 10A–C). Maxillule with one large setae at upper margin and cutting edge divided into three zig-zagged portions, each portion three to four large setae; interior margin slightly convex (Figure 10D–H). Mandibles penta-dentoid; first teeth large, robust and sharp, separated from the remainders (Figure 11A–D). The fifth teeth are very small, 4–5 times smaller than the third and fourth teeth (Figure 11C,D). Size of fifth teeth comparable to the size of the inferior angle (Figure 11D). Mandibular palp elongated and with serrulate setae on tip and outer margin (Figure 11E,F). Labrum not bullate, with a small, concaved notch; a single array of small sharp teeth at notch (Figure 11G,H).

**Distribution:** Cosmopolitan.

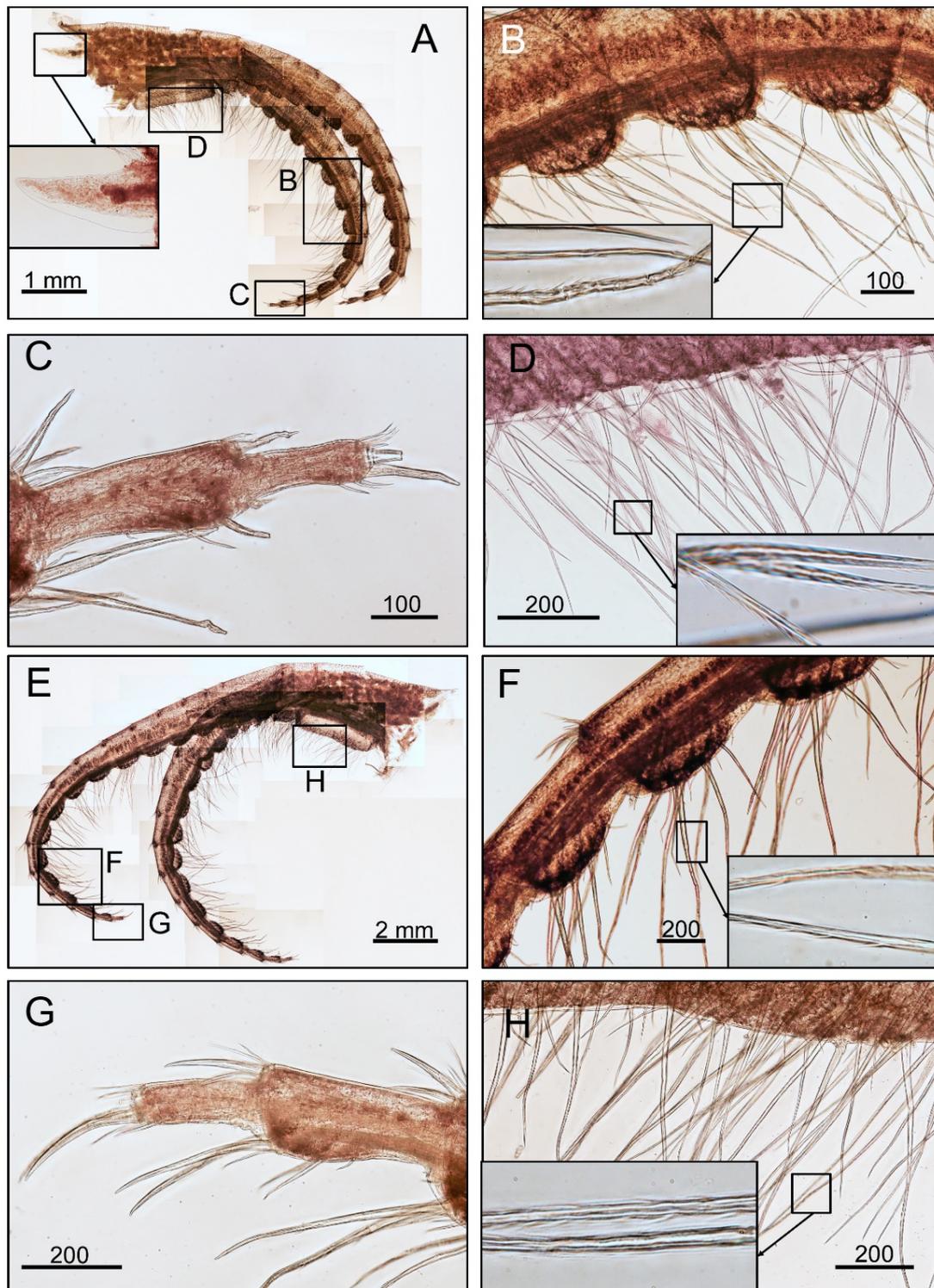
**Remarks:** There are consistent morphological differences between *C. virgatum* and *C. hunteri*. The bending of the tergum and size of the fifth teeth of the mandible are consistently diagnostic between the two species. *C. hunteri* has a bended tergum, and its fifth tooth in the mandible is much smaller than that of *C. virgatum* (Figure 12).

### 3.2. Molecular Analysis

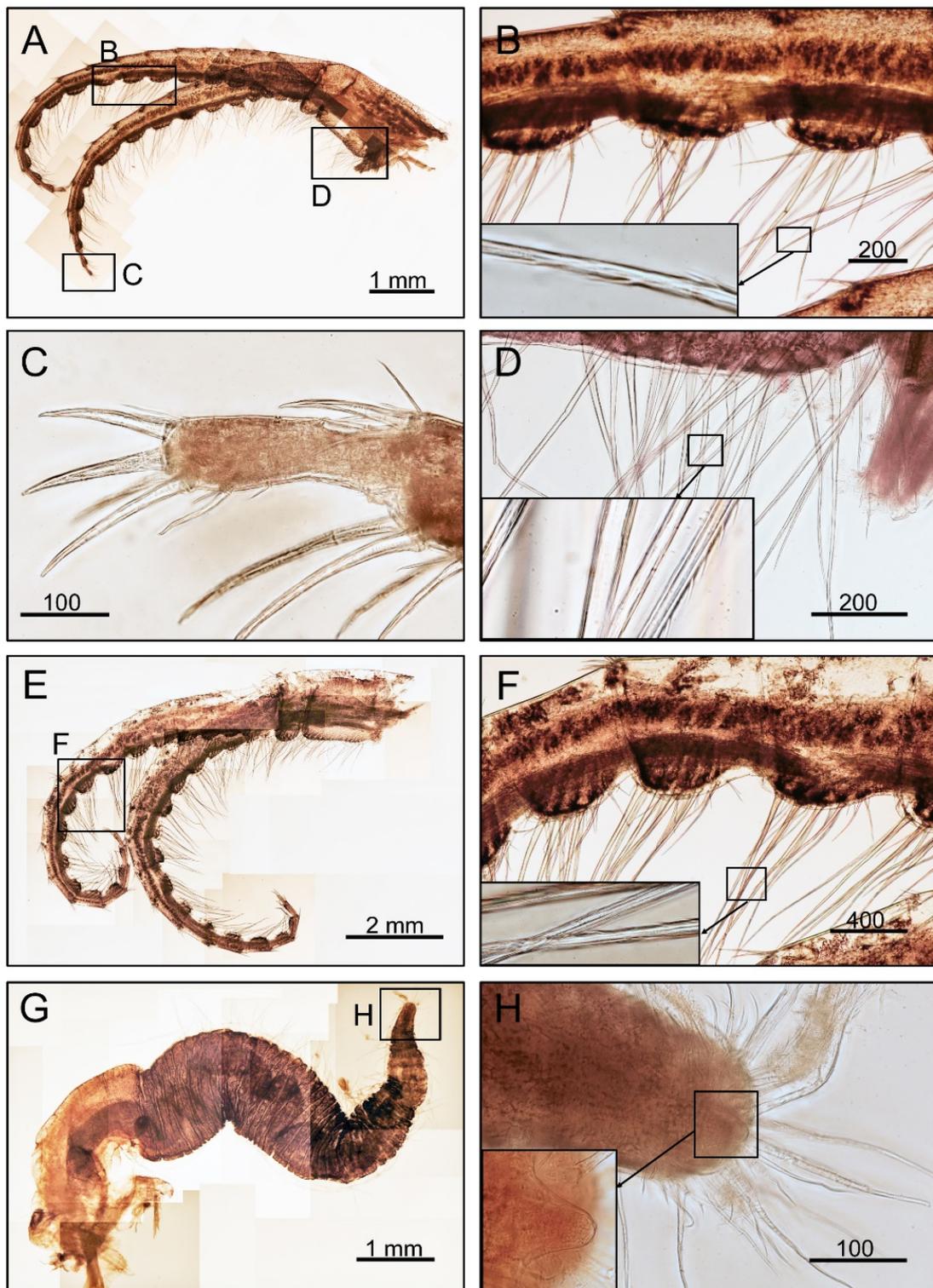
All the phylogenetic results suggested that both *Conchoderma hunteri* and *C. virgatum* were clustered into their own clades with high bootstrap values and posterior probabilities support, except for the BI, which the posterior probability for *C. virgatum* cluster was 67.43 (Figure 13). Both species form a sister group and *C. auritum* has the sister relationship with *C. hunteri* and *C. virgatum* in the NJ and ML results (Figure 13). However, the BI analysis showed polytomy with *C. hunteri* + *C. virgatum*, *C. auritum*, and *L. australis* + *L. anserifera* (Figure 13).



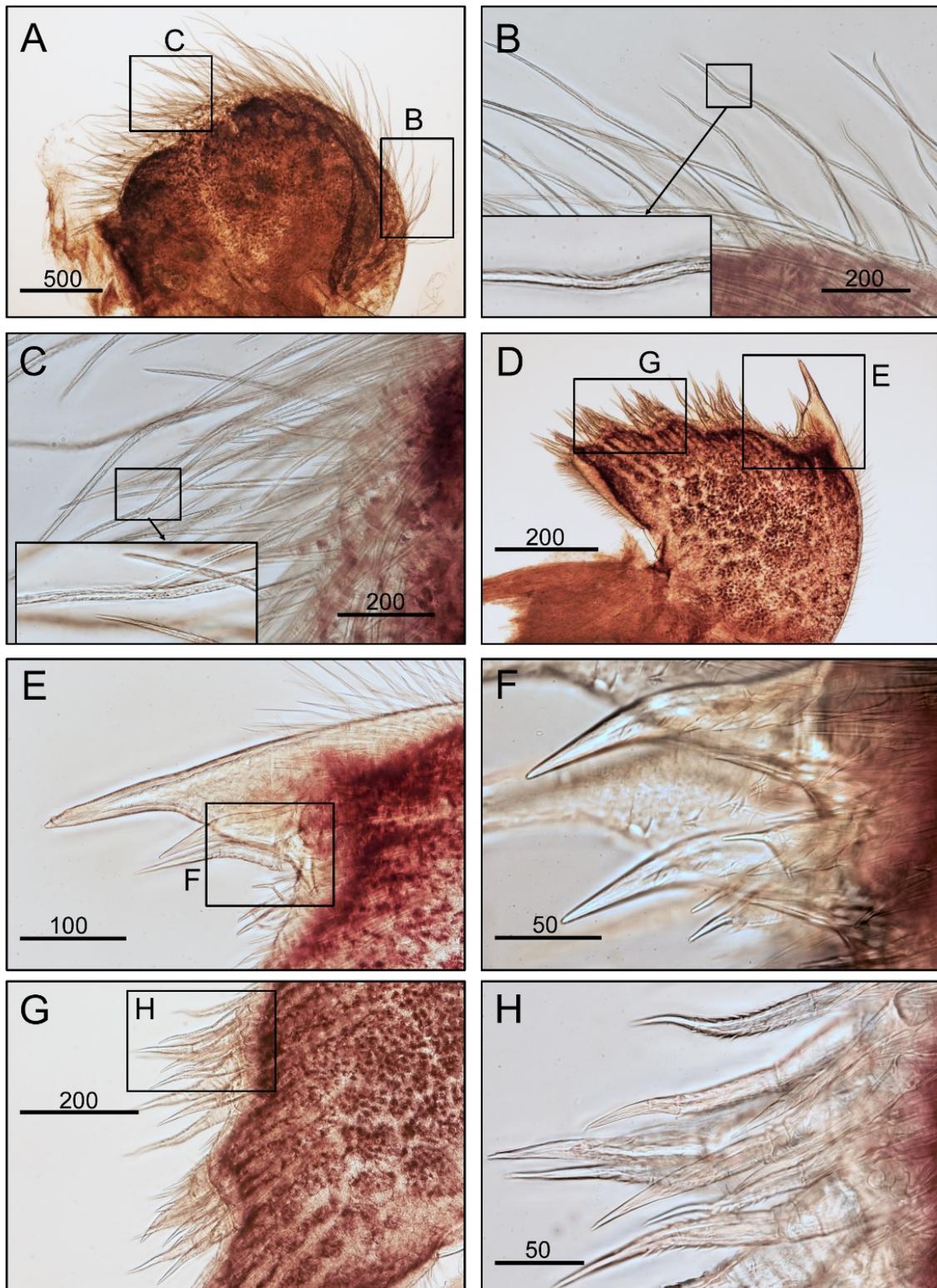
**Figure 7.** *Conchoderma hunteri*. (A) Cirrus I, showing two filamentary appendages at base region. (B) Posterior ramus, showing serrulate setae. (C) Anterior ramus, showing serrulate setae. (D) Pro-tuberant segment of cirrus I, showing serrulate setae. (E) Cirrus II. (F) Posterior ramus showing serrulate setae. (G) Anterior ramus, showing serrulate setae. (H) Intermediate segment of anterior ramus, showing serrulate setae. Scale bars in  $\mu\text{m}$ .



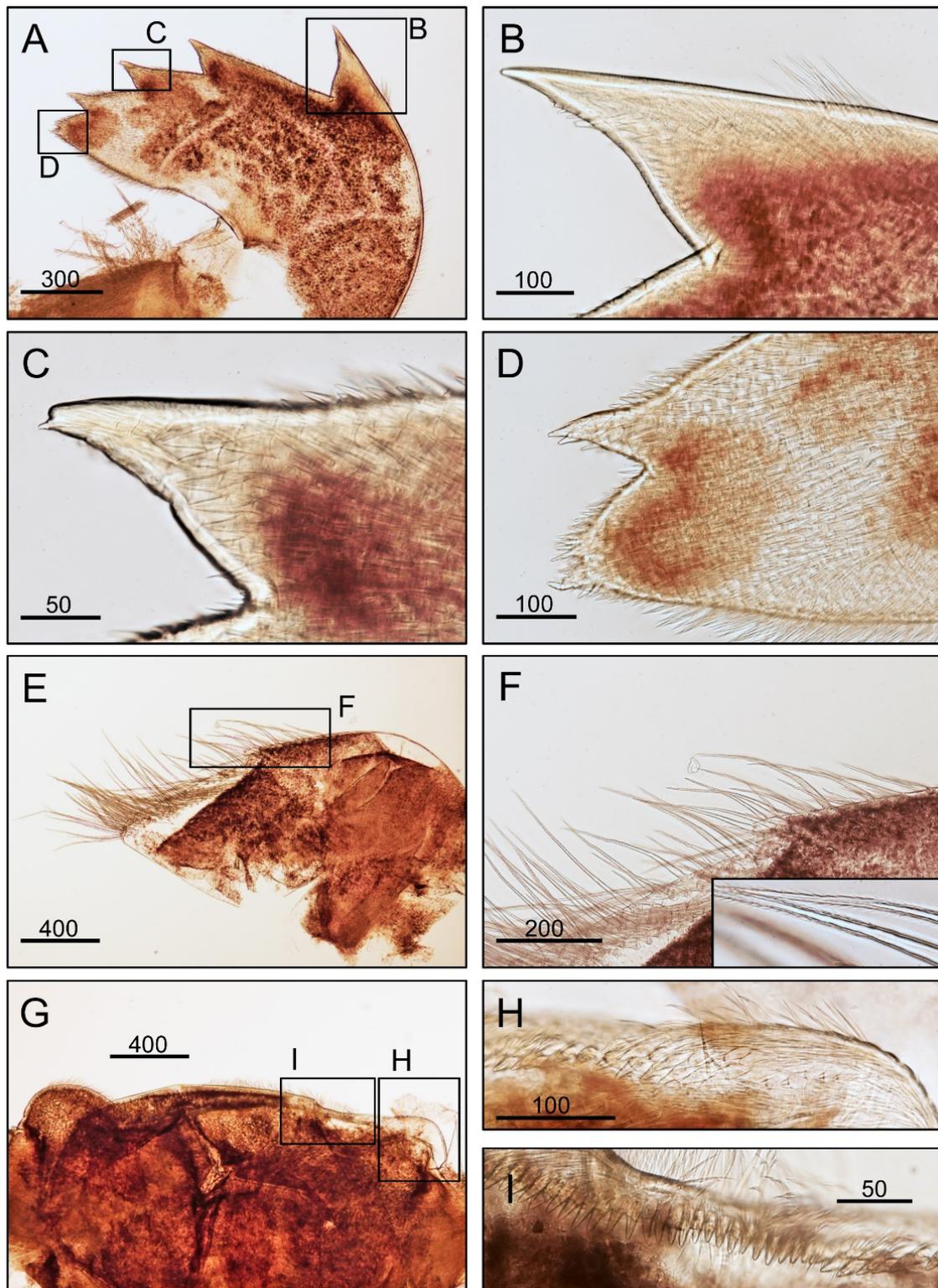
**Figure 8.** *Conchoderma hunteri*. (A) Cirrus III, note single filamentary appendage at base. (B) Intermediate segments of posterior ramus, showing serrulate setae. (C) Distal segment of posterior ramus. (D) Magnified segment of anterior ramus of cirrus III, showing serrulate setae. (E) Cirrus IV. (F) Serrulate-type setae at cirrus IV. (G) Distal end of posterior ramus of cirrus IV, showing short robust simple setae. (H) Intermediate segment of cirrus IV, showing serrulate setae. Scale bars in  $\mu\text{m}$ .



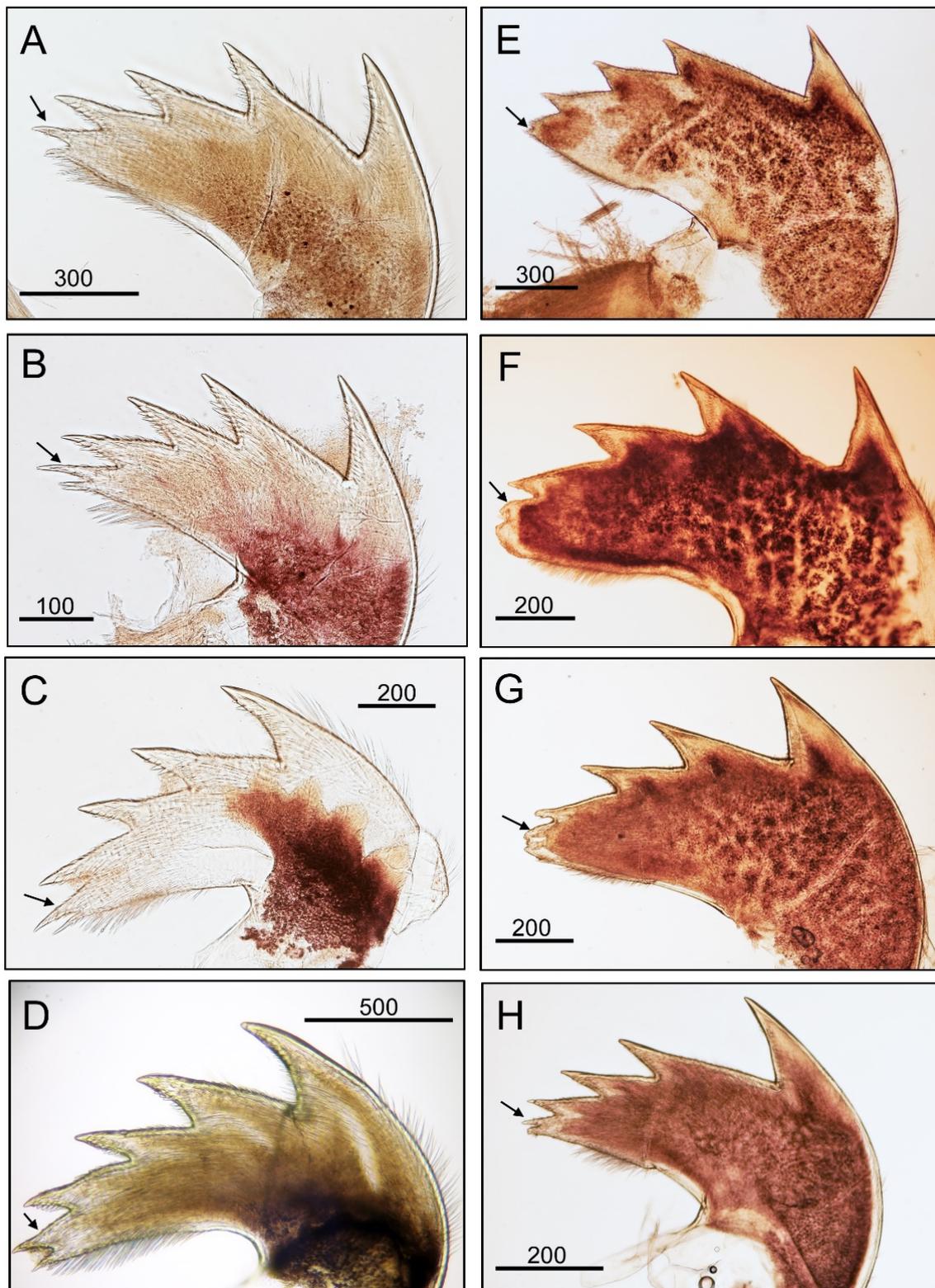
**Figure 9.** *Conchoderma hunteri*. (A) Cirrus V. (B) Intermediate segment of cirrus V showing serrulate setae. (C) Distal segment of cirrus V, showing robust simple setae. (D) Intermediate segment of cirrus V showing serrulate setae. (E) Cirrus VI. (F) Serrulate setae at intermediate segment of cirrus VI. (G) Penis. (H) Apex of penis. Scale bars in  $\mu\text{m}$ .



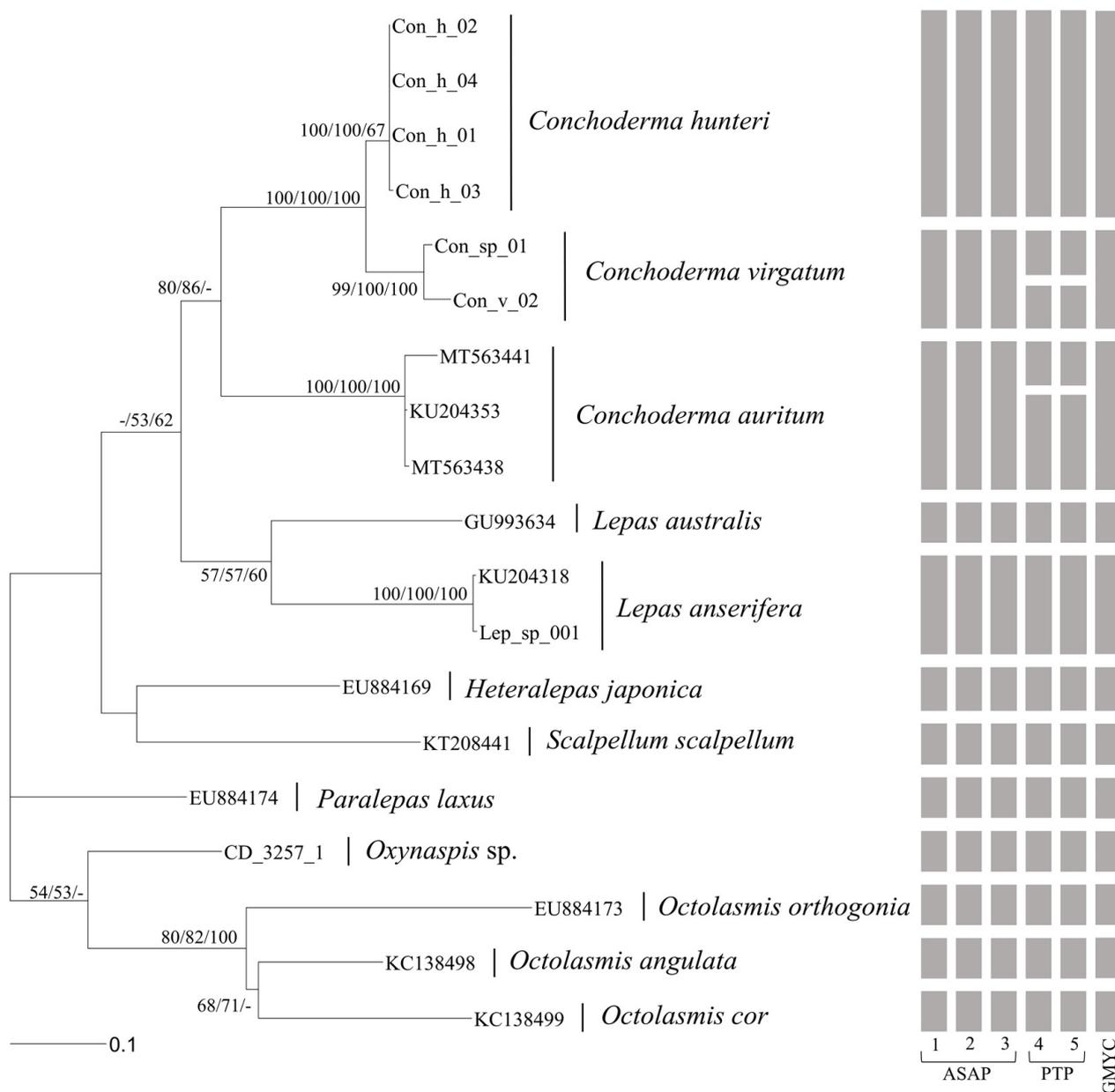
**Figure 10.** *Conchoderma hunteri*. (A) Maxilla. (B) Serrulate setae at outer margin of maxilla. (C) Serrulate setae at inner margin of maxilla. (D) Maxillule, showing three zig-zag regions at cutting edge. (E) Large spine of maxillule. (F) Simple setae at the base of large spine of maxillule. (G) Cutting edge of maxillule. (H) Serrulate-type setae at cutting edge of maxillule. Scale bars in  $\mu\text{m}$ .



**Figure 11.** *Conchoderma hunteri*. (A) Mandible, showing five teeth. (B) First tooth of mandible. (C) 3rd tooth of the mandible. (D) Small 5th tooth of the mandible, size comparable to the inferior angle. (E) Mandibular palp. (F) Serrulate setae at margin of palp. (G) Labrum. (H) Cutting edge of labrum showing small teeth. Scale bars in  $\mu\text{m}$ .



**Figure 12.** Comparison of mandible from four different specimens of *C. virgatum* (A–D) and four different specimens of *C. hunteri* (E–H). Note consistent differences in the size of 5th tooth (indicated by arrows) between *C. virgatum* and *C. hunteri*. Scale bars in μm.



**Figure 13.** A Maximum likelihood (ML) phylogeny tree reconstructed with COI sequences by using MEGA11. Bootstrap values of neighbor joining (NJ), maximum likelihood (ML), and posterior probability of Bayesian inference (BI) are shown at the nodes when >50%, respectively. The species delimitation results of ASAP, PTP, and GMYC are indicated with vertical bars. The number of ASAP represent 3 models used for analysis, (1) Jukes-Cantor model (JC69), (2) Kimura 2-parameter model (K2P), and (3) Simple distance ( $p$ -distances). The number of PTP analysis represent (4) Bayesian Poisson tree processes (bPTP) and (5) the PTP result of the Maximum likelihood solution.

The K2P distances within *C. hunteri* and *C. virgatum* were  $0.002 \pm 0.001$  and  $0.034 \pm 0.008$  for the COI sequences, respectively. The K2P distances between *C. hunteri* and *C. virgatum* were  $0.097 \pm 0.016$ . The K2P distances between these two species and other species ranged from 0.242 to 0.354 (Table 2).

**Table 2.** Kimura 2-parameter (K2P) distances of COI sequences between species using MEGA X. The lower left of the matrix are the mean distances, and the upper right of the matrix are the SD. Bolded values indicate the comparison between *C. hunteri* and *C. virgatum*.

	1	2	3	4	5	6	7	8	9	10	11	12
1. <i>Conchoderma hunteri</i>		<b>0.016</b>	0.032	0.035	0.035	0.036	0.038	0.036	0.036	0.041	0.045	0.044
2. <i>Conchoderma virgatum</i>	<b>0.097</b>		0.030	0.035	0.042	0.034	0.036	0.036	0.038	0.042	0.041	0.040
3. <i>Conchoderma auritum</i>	0.257	0.242		0.039	0.039	0.043	0.044	0.042	0.040	0.049	0.044	0.046
4. <i>Lepas australis</i>	0.278	0.285	0.300		0.032	0.033	0.042	0.034	0.039	0.042	0.042	0.039
5. <i>Lepas anserifera</i>	0.276	0.339	0.313	0.257		0.035	0.051	0.039	0.044	0.043	0.044	0.044
6. <i>Heteralepas japonica</i>	0.288	0.282	0.345	0.258	0.278		0.031	0.036	0.035	0.037	0.041	0.038
7. <i>Salpella scalpellum</i>	0.311	0.298	0.346	0.334	0.396	0.255		0.035	0.036	0.039	0.040	0.036
8. <i>Paralepas laxus</i>	0.279	0.281	0.336	0.264	0.320	0.278	0.278		0.031	0.042	0.031	0.039
9. <i>Oxyropsis sp.</i>	0.278	0.306	0.320	0.307	0.353	0.277	0.281	0.231		0.035	0.031	0.035
10. <i>Octolasmis orthogonia</i>	0.321	0.336	0.394	0.337	0.346	0.307	0.315	0.350	0.275		0.030	0.033
11. <i>Octolasmis angulata</i>	0.350	0.333	0.363	0.331	0.343	0.330	0.321	0.248	0.235	0.236		0.029
12. <i>Octolasmis cor</i>	0.354	0.331	0.399	0.304	0.358	0.313	0.288	0.306	0.268	0.272	0.206	

Generally, the three morphospecies of *Conchoderma* were always in different MOTUs (Molecular Operational Taxonomic Units). However, PTP analyses further split *C. hunteri* and *C. virgatum* into two MOTUs each.

#### 4. Discussion

The present study revealed that *Conchoderma virgatum* and *C. hunteri* are different species based on morphological and molecular evidence, rejecting the hypothesis of their conspecificity proposed by Hiro (1937) [13] and Zevina (1982) [18]. First of all, the species have different terga. The tergum of *C. hunteri* bends at a 1/4 position of the distal region. The tergum of *C. virgatum* is relatively straight and does not bend. The distal one-third portion of the carina overlaps with the tergum in *C. hunteri*. Mandibles of *C. hunteri* have small fifth teeth, in contrast to the bigger fifth teeth in *C. virgatum*. There are no diagnostic differences between their somatic bodies—both have a similar number and position of paired filamentary appendages, cirri, and mouth parts (except the mandible).

Based on our molecular analysis, *Conchoderma virgatum* and *C. hunteri* form sister clades with high support, indicating that the two species are closely related, and these two species also share similar epibiotic habitats [2–6] and similar morphology (cirri and filamentary appendages on somatic bodies). The phylogeny analysis and specimen delimitation result of ASAP and GMYC all suggested that *C. virgatum* and *C. hunteri* are different species. The results of PTP also divide *C. virgatum* and *C. hunteri* into two groups. However, PTP further indicated that *C. virgatum* might have two groups, which need further examination on morphological features from larger sample sizes to confirm.

The present study confirmed that there are currently five species in *Conchoderma*—*C. virgatum*, *C. hunteri*, *C. auritum*, *C. indicum*, and *C. chelonophilum* which differ in their number of filamentary appendages, shape of their tergum, and morphology of their capitulum. Darwin [7] considered *C. chelonophilum* to be a variety of *C. virgatum* that lives exclusively on turtles. Hoek [16] redescribed *C. chelonophilum* and doubted that it is its own species based on its exclusively occurrence on sea turtles, smaller size, and different capitulum shape compared to *C. virgatum*. Due to high morphological variations between *C. chelonophilum* and *C. virgatum*, Hoek [16] considered *C. chelonophilum* to be a subspecies of *C. virgatum*. Jones [72] considered *C. chelonophilum* to be a distinct species. A further record of *C. virgatum chelonophilum* is from loggerhead turtles in Australia [61]. A more recent record of *C. virgatum* is from sea turtles in Uruguay [64], but that publication contained photographs of a colony of *Conchoderma* on the turtles without detailed morphological examinations. Those specimens from turtles in Uruguay probably represents *C. chelonophilum*.

Moving forward, the taxonomic status of *C. chelonophilum* should be further evaluated using morphological and molecular analyses.

Key to species of *Conchoderma*

- (1) With ear-like extension in the capitulum . . . . . *Conchoderma auritum*
- (2) Without ear-like extension in capitulum . . . . . (3)
- (3) 2nd cirri with filamentary appendages . . . . . *Conchoderma indicum*
- (4) 2nd cirri without filamentary appendages . . . . . (5)
- (5) Tergum bended at 1/3 distal portion . . . . . *Conchoderma hunteri*
- (6) Tergum not bended . . . . . (7)
- (7) Live on sea turtles . . . . . *Conchoderma chelonophilum*
- (8) Live on other marine substratum/organism . . . . . *Conchoderma virgatum*

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d14080593/s1>. Aligned sequences with with Clustal W in MEGAX.

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